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Arsenic Directly Binds to and Activates the Yeast AP-1-Like Transcription Factor Yap8

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The AP-1-like transcription factor Yap8 is critical for arsenic tolerance in the yeast *Saccharomyces cerevisiae*. However, the mechanism by which Yap8 senses the presence of arsenic and activates transcription of detoxification genes is unknown. Here we demonstrate that Yap8 directly binds to trivalent arsenite [As(III)] *in vitro* and *in vivo* and that approximately one As(III) molecule is bound per molecule of Yap8. As(III) is coordinated by three sulfur atoms in purified Yap8, and our genetic and biochemical data identify the cysteine residues that form the binding site as Cys132, Cys137, and Cys274. As(III) binding by Yap8 does not require an additional yeast protein, and Yap8 is regulated neither at the level of localization nor at the level of DNA binding. Instead, our data are consistent with a model in which a DNA-bound form of Yap8 acts directly as an As(III) sensor. Binding of As(III) to Yap8 triggers a conformational change that in turn brings about a transcriptional response. Thus, As(III) binding to Yap8 acts as a molecular switch that converts inactive Yap8 into an active transcriptional regulator. This is the first report to demonstrate how a eukaryotic protein couples arsenic sensing to transcriptional activation.

Arsenic is a highly toxic metalloid that is widespread in the environment. Consequently, nearly all organisms have developed sophisticated strategies for arsenic detoxification and tolerance acquisition (1). These mechanisms include increased export from cells, sequestration in organelles, chelation by metal-binding proteins and peptides, and diminished uptake. Many detoxification systems are controlled at the transcriptional level by regulatory proteins that are activated during arsenic exposure (2–5). The best-studied arsenic-responsive regulator is the *Escherichia coli* ArsR repressor protein (6). In untreated cells, ArsR is bound to DNA and represses transcription of arsenic tolerance genes. Upon binding of trivalent arsenite to vicinal cysteine residues, ArsR dissociates from the promoter, resulting in induced gene expression (7). ArsR-type repressors are widespread in bacteria and archaea but are absent in higher eukaryotes. In fact, very little is known about arsenic-sensing regulators in eukaryotes.

Global gene expression analyses and gene-specific studies have implicated several proteins that might mediate the transcriptional response to arsenic in the eukaryotic microbe *Saccharomyces cerevisiae* (budding yeast) (2, 8). Two of these transcriptional regulators are Yap1 and Yap8 (also called Acr1 and Arr1). Both proteins belong to the yeast AP-1 family of transcription factors and contain conserved basic leucine-zipper (bZIP) DNA binding domains and conserved cysteine residues (9). Yap1 controls expression of genes encoding functions in oxidative stress defense and sulfur metabolism in response to arsenite exposure (10–12). Yap8 has a highly specialized function and regulates expression of only two genes: *ACR2*, encoding an arsenate [As(V)] reductase, and *ACR3*, encoding an arsenite [As(III)] and antimonite [Sb(III)] exporter. These genes are transcribed in opposite directions from a common promoter that contains a 13-bp element to which Yap8 binds (12–15). *ACR2* and *ACR3* expression is induced ~20- to 30-fold when cells are exposed to As(III). Cells that lack *YAP8* cannot induce *ACR2* and *ACR3* expression and are sensitive to As(V), As(III), and Sb(III). Likewise, deletion or alteration of the DNA sequence to which Yap8 binds results in diminished *ACR2/ACR3* expression and arsenic sensitivity (12, 15). These data firmly establish Yap8 as a critical regulator of arsenic detoxification genes in *S. cerevisiae*. However, the mechanism by which Yap8 senses the presence of metalloids and activates gene expression is not understood.

Arsenic may affect proteins by forming metal-thiol bonds with vicinal cysteines or by causing oxidative modifications of amino acid side chains (16, 17). ArsR repressors typically have three cysteine residues that form the As(III) binding site (18–20). Yap8 has eight cysteines, three of which, Cys132, Cys137, and Cys274, are conserved in several fungal AP-1 proteins (9). Mutation of any of these three conserved cysteines (Cys132, Cys137, Cys274) results in the inability of Yap8 to induce *ACR2/ACR3* expression and to confer arsenic tolerance (12, 21, 22). Yap8 resides in the nucleus, where it constitutively binds to the *ACR2/ACR3* promoter as a homodimer. Neither the presence of As(III) nor mutation of the three critical cysteines affects the nuclear localization of Yap8 (12, 21). We previously observed that Yap8 is regulated at the post-translational level; in untreated cells, Yap8 levels are low due to degradation via the ubiquitin-proteasome system, whereas in As(III)-treated cells, Yap8 escapes degradation and its protein levels are increased. This As(III)-induced stabilization of Yap8 required the three critical cysteines. However, the purpose of this

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regulation remained unclear since a dramatic increase in Yap8 protein levels only moderately enhanced *ACR3* expression. In contrast to what occurs with As(III) exposure, Yap8 levels were not affected in cells exposed to oxidative stress agents (peroxide and paraquat) or in cells that overexpressed enzymes that protect cells from oxidative damage (catalase, superoxide dismutase) (21), suggesting that Yap8 might not be regulated by oxidative protein modifications. The observations that Cys132, Cys137, and Cys274 are each required for As(III)-induced Yap8 stabilization (21), for Yap8-dependent induction of *ACR2* and *ACR3* expression, and for arsenic resistance (12, 21, 22) implicate them in arsenic sensing. Here, we demonstrate that Yap8 senses arsenic by direct binding to the three conserved cysteine residues. As(III) binding triggers a conformational change in Yap8 that induces transcription of *ACR2* and *ACR3*.

MATERIALS AND METHODS

Yeast strains and growth conditions. *S. cerevisiae* strains W303-1A (*MAT***a** *ura3-1 trp1-1 leu2-3/112 his3-11/15 ade2-1 can1-100*) (23) and RW117 (W303-1A *yap8*Δ::*kanMX*) (12) were cultivated in minimal YNB (0.67% yeast nitrogen base) medium supplemented with auxotrophic requirements and 2% glucose as carbon source. Sodium arsenite (Sigma) was added directly to the growth medium, and plate growth assays were performed as previously described (24).

Protein expression and purification. Plasmids expressing N-terminal fusions of Yap8 and Yap8-C132A/C274A to maltose-binding protein (MBP) were constructed by inserting the corresponding coding DNA fragments into plasmid pMAL-c2x (New England BioLabs) between the BamHI and HindIII sites. Cells of *E. coli* strain BL21(DE3) transformed with the expression plasmids were grown in 2 ml LB medium overnight at 37°C and used to inoculate 1 liter of the same medium on the next day. When the A_{600} of the culture reached 0.6 to 0.8 at 37°C, the cell culture was cooled to 16°C, and protein expression was induced by addition of 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) overnight at 16°C. MBP-Yap8 and MBP-Yap8-C132A/C274A proteins were purified as previously described (25). Purified proteins were stored at −80°C until use, and their concentrations were determined according to the method of Bradford (26) or from the absorption at 280 nm (27).

XAS. X-ray absorption spectroscopy (XAS) data were collected at the National Synchrotron Light Source (NSLS) on beam line X3B using an Si (111) crystal monochromator equipped with a harmonic rejection mirror. Samples were maintained at 25 K using an Oxford Instruments continuous-flow liquid helium cryostat. Protein fluorescence excitation spectra were collected using a Canberra 30-element Ge solid-state array detector. A germanium filter (0.3 μm in width) was placed between the cryostat and detector to filter scattering fluorescence not associated with protein arsenic signals. XAS spectra were measured using 5-eV steps in the pre-edge region (11,625 to 11,825 eV), 0.3-eV steps in the edge region (11,850 to 11,900 eV), and 0.05-$Å^{-1}$ increments in the extended X-ray absorption fine structure (EXAFS) region out to a photoelectron vector (k) value of 13 $Å^{-1}$, integrating from 2 to 20 s in a k-weighted manner in the EXAFS region for a total scan length of 45 min. X-ray energies were calibrated using an arsenic foil absorption spectrum collected simultaneously with the protein data. The first inflection point for the arsenic foil edge was assigned to 11,867 eV. Each fluorescence channel of each scan was examined for spectral anomalies prior to averaging, and spectra were closely monitored for photodamage. The spectral figures presented represent the average of 12 to 14 scans. XAS data were processed using the Macintosh OS X version of the EXAFSPAK program suite (28) integrated with the Feff v8 software (29) for theoretical model generation. Data reduction followed a previously published protocol for a spectral resolution in bond length of 0.13 Å (30). EXAFS fitting analysis was performed on raw/unfiltered data. Protein EXAFS data were fit using single-scattering Feff theoretical models calculated for carbon, oxygen, and sulfur coordination to simulate arsenic-ligand environments, with values for the scale factors (Sc) and E_0 calibrated by fitting crystallographically characterized arsenic model compounds, as previously outlined (31). Criteria for judging the best-fit EXAFS simulations utilized both the lowest mean square deviation between data and the fit corrected for the number of degrees of freedom (F') (32) and reasonable Debye-Waller factors ($\sigma^2 < 0.006$ $Å^2$) (33).

Metalloid binding assays. The buffer of purified MBP-Yap8 and MBP-Yap8-C132A/C274A was exchanged with a buffer containing 50 mM morpholinepropanesulfonic acid (MOPS)-KOH, pH 7, using a Bio-Gel P-6 Micro Bio-Spin column (Bio-Rad). The buffers were degassed by bubbling with argon. Then, wild-type and mutant forms of Yap8 were incubated at 4°C with the indicated concentrations of Sb(III) in the form of potassium antimonyl tartrate (Sigma) or As(III) in the form of sodium arsenite (Sigma). After 1 h, each sample was passed through a Bio-Gel P-6 column preexchanged with the same buffer to remove the free metalloid. A portion of the eluate was diluted with 2% HNO_3, and the quantity of metalloid was measured by inductively coupled mass spectrometry (ICP-MS) with a PerkinElmer Elan 9000. Antimonite and arsenite standard solutions in the range of 0.5 to 10 ppb in 2% HNO_3 were obtained from Ultra Scientific Inc. (North Kingstown, RI).

DNA binding by fluorescence anisotropy. DNA binding studies by fluorescence anisotropy were performed as previously described (19) using a Photon Technology International spectrofluorimeter fitted with polarizers in the L format. Complementary 31-mer oligonucleotides, one of which was labeled at the 5′ end with carboxyfluorescein (FAM), were synthesized (Integrated DNA Technologies Inc.) based on the reported Yap8 binding site (indicated in bold): 6-FAM-5′-CTTTTTGTT**TGATTAATAATCA**ACTTTAGCG-3′ and 5′-CGCTAAAGT**TGATTATTAATCA**AACAAAAAG-3′ (15). Desalted DNA oligonucleotides were heated at 94°C for 5 min, annealed by cooling to room temperature, and stored in aliquots at −20°C. The change of anisotropy of 10 nM DNA was monitored after addition of different amounts of purified MBP-Yap8 or MBP-Yap8-C132A/C274A in a buffer consisting of 50 mM MOPS-NaOH, pH 7.5, 0.2 M NaCl, and 5 mM Tris(2-carboxyethyl)phosphine (TCEP), in the presence or absence of 5 mM As(III).

As-biotin assay. Arsenic was tagged with a biotin label as previously described (34) to allow pulldown of arsenic binding proteins with streptavidin-agarose beads. Plasmids encoding N-terminal fusions of Yap8 and the Yap8-C132A/C274A mutant to glutathione *S*-transferase (GST) were constructed by inserting the corresponding DNA fragments into plasmid pGEX4T-1 (GE Healthcare) as described previously (15). *E. coli* BL21(DE3) cells expressing GST-Yap8 and GST–Yap8-C132A/C274A were incubated for 4 h at 30°C with 1 mM IPTG and lysed by sonication in a buffer containing 100 mM Tris (pH 8), 10% glycerol, 5 mM TCEP, and protease inhibitor cocktail (Roche). One hundred micrograms of the bacterial lysates was incubated with 10 μM As-biotin conjugate (see Fig. 2) at 30°C for 30 min. In some cases, lysates were pretreated with 10 μM As(III) for 30 min prior to the addition of conjugate. The cell lysate was then diluted 10 times with urea buffer (10 mM Tris [pH 8], 100 mM Na_2HPO_4, 100 mM NaCl, 10% glycerol, 0.1% NP-40, 4 M urea), mixed with 50 μl of streptavidin-agarose resin, and shaken gently at 4°C for 2 h. The resin was briefly spun down, washed twice with urea buffer, and resuspended in 50 μl of 1× SDS loading buffer containing 0.4 M urea for SDS-PAGE. After Western blotting, GST-tagged Yap8 was detected using an anti-GST antibody (G1160; Sigma). The same procedure was followed for GST-tagged *Kl*Yap8 from *Kluyveromyces lactis* (35) with the exception that 50 μg of bacterial lysates was incubated with the As-biotin conjugate.

For assays in yeast, plasmids encoding myc-Yap8 (21) or myc-Yap8-C132A/C274A (this study) were transformed into *yap8*Δ cells, and the transformants were grown to mid-logarithmic phase. Two hundred micrograms of yeast lysates was incubated with 10 μM As-biotin conjugate, followed by the procedure described above. To detect myc-tagged Yap8, an anti-c-myc antibody (clone 9E10; Roche) was used.

Trypsin susceptibility assay. *E. coli* cells expressing GST-Yap8 and GST–Yap8-C132A/C274A were induced for 4 h at 30°C with 1 mM IPTG and then treated with or without 0.5 mM As(III) for 45 min before collection. Cells were washed, resuspended in phosphate-buffered saline (PBS) buffer containing EDTA-free protease inhibitor cocktail (Roche), followed by sonication to lyse the cells. Ten micrograms of bacterial lysates was incubated with trypsin (Sigma) at the indicated concentrations for 10 min on ice before addition of 0.5 μg/ml soybean trypsin inhibitor (Sigma) for an additional 15 min on ice. Proteolysis patterns were visualized after Western blotting using an anti-GST antibody (G1160; Sigma) and quantified using the Quantity One software (Bio-Rad).

For assays in yeast, plasmids encoding myc-Yap8 or myc-Yap8-C132A/C274A were transformed into *yap8*Δ cells and grown until mid-logarithmic phase. Cells were either left untreated or exposed to 0.5 mM As(III) for 1 h. After lysing the cells in PBS buffer using glass beads, the same procedure as that described above was followed, with the exception that 100 μg of yeast lysates was incubated with trypsin. Proteolysis patterns were visualized after Western blotting using an anti-c-myc antibody (clone 9E10; Roche) and quantified as above.

Fluorescence microscopy. To follow the distribution of green fluorescent protein (GFP)-tagged fusion proteins, transformants expressing GFP-Yap8 and GFP–Yap8-C132A/C274A (12) were grown in YNB medium lacking the appropriate amino acid to mid-log phase. Cells were washed twice with water or PBS, and GFP signals were observed in living cells before and 10 min after exposure to 1 mM As(III) using a Leica DM R fluorescence microscope. Images were captured with a digital camera (Hamamatsu C4742-95; Hamamatsu Photonics) and QFluoro software and processed with Photoshop CS (Adobe Systems).

qPCR. *yap8*Δ cells expressing GFP-Yap8 or GFP–Yap8-C132A/C274A were grown overnight at 30°C in YNB medium with appropriate selection. The overnight cultures were inoculated to fresh medium at an optical density at 600 nm (OD_{600}) of 0.4 and grown until the exponential phase at 30°C. After taking a null sample and exposing the cells to 0.5 mM As(III), samples were collected at the indicated time points. Total RNA was extracted from the samples with the RNeasy minikit (Qiagen), converted to cDNA (SuperScript II-Reverse Transcriptase; Invitrogen), and used for quantitative real-time PCR (qPCR) analyses. We used the iQ SYBR green supermix (Bio-Rad) for the reactions and designed the primers using Beacon Designer 4.25. The qPCRs were carried out with an iQ5 Multicolor real-time PCR thermal cycler (iCycler; Bio-Rad) and the iQ5 (version 2.1) software. *IPP1* was used as the reference gene and for normalizing all reactions. Reactions were run in triplicates, and mean values were taken. The comparative threshold cycle (C_T) method for relative quantification ($\Delta\Delta C_T$ method), which describes the change in expression of the target genes in a test sample relative to a calibrator sample, was used to analyze the data.

ChIP. Chromatin immunoprecipitation (ChIP) was performed largely as previously described (36). Volumes of 100 ml of mid-log-phase cells were divided into two halves that were left untreated or exposed to 0.5 mM As(III) for 1 h and then cross-linked (1% formaldehyde, 15 min, 30°C) followed by neutralization (150 mM glycine, 5 min). Cell pellets were washed twice in cold Tris-buffered saline (TBS; 0 mM Tris-HCl [pH 7.5] and 150 mM NaCl), resuspended in 600 μl cold FA-lysis buffer (50 mM HEPES-KOH [pH 7.5], 140 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate, protease inhibitor cocktail; Roche), and then lysed at 4°C using glass beads. After glass bead removal, samples were centrifuged (14,000 rpm, 4°C, 15 min) and the supernatant was sonicated to shear DNA to an average size of 500 bp. An additional 400 μl of FA-lysis buffer was added, and the chromatin lysate was purified by centrifugation (14,000 rpm, 1 h, 4°C). For immunoprecipitations (IPs), 300 μl of the purified chromatin lysate was mixed with 2.5 μl anti-c-myc antibody (9E10; Roche). All IP mixtures were incubated overnight at 4°C, followed by incubation with 10 to 15 μl equilibrated protein G Dynabeads (Life Technologies) for 4 h at 4°C. Beads were washed at room temperature, for 10 min each, sequentially with FA-lysis buffer, FA-500 buffer (50 mM HEPES-KOH [pH 7.5], 500 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate), LiCl wash buffer (10 mM Tris-HCl [pH 8.0], 250 mM LiCl, 1 mM EDTA, 0.5% NP-40, 0.5% sodium deoxycholate), and Tris-EDTA (TE; 10 mM Tris-HCl [pH 7.5], 1 mM EDTA), followed by elution in elution buffer (50 mM Tris-HCl [pH 7.5], 10 mM EDTA, 1% sodium dodecyl sulfate) with shaking for 10 min at 65°C. For input samples, 20 μl purified chromatin lysate was diluted in 300 μl TE. All samples (IPs and inputs) were treated with RNase (0.1 μg/ml), and cross-links were reversed (65°C overnight) followed by treatment with proteinase K (0.2 mg/ml, 42°C, 2 h). DNA was purified using a kit (Zymo research), and the IP and input DNA was analyzed by qPCR. The percentage (Input %) value for each sample was calculated as follows: ΔC_T [normalized ChIP] = C_T [ChIP] − {C_T [Input] − $\log_2$ (dilution factor)} and Input % = $100/2^{\Delta CT\ [\text{normalized ChIP}]}$. The "Input %" value represents the enrichment of protein at the *ACR3* promoter and is normalized to a control region of the *IPP1* gene.

RESULTS

Purified Yap8 binds to As(III) via three cysteine residues. To test the possibility that Yap8 directly binds arsenic, we first fused Yap8 to the C-terminal end of maltose binding protein (MBP), which does not bind As(III), expressed the fusion protein in *E. coli*, and purified MBP-Yap8 with an amylose resin. Purified MBP-Yap8 was incubated with As(III), unbound As(III) was removed, and the nature of the binding site was investigated. X-ray absorption near edge structure (XANES) analysis indicated that arsenic is stably bound as As(III) in MBP-Yap8 (Fig. 1A). The observed value for the first inflection point energy is 11,868 eV, consistent with observed values for As(III) in protein and model systems (31). Extended X-ray absorption fine structure (EXAFS) analysis for MBP-Yap8 is consistent with a three-coordinate As(III)-nearest neighbor coordination system constructed by sulfur-based ligands only at 2.24 Å (Fig. 1B; Table 1). The EXAFS analysis also shows long-range carbon interaction at 2.7 Å and 3.69 Å (Fig. 1B; Table 1). Since there was no thiol present in the buffers, these data indicate that the As(III) binding site in Yap8 is formed by three cysteine residues.

We next measured binding of As(III) to purified MBP-Yap8 as a function of As(III) concentration by rapid gel filtration to separate free metalloid from apo-Yap8 and Yap8-As(III) complex. Purified MBP-Yap8 bound As(III) with a stoichiometry of approximately one arsenite molecule per molecule of Yap8 (Fig. 1C). Since Yap8 has been implicated in the cellular response to antimonite (12, 13), we also measured Sb(III) binding. MBP-Yap8 bound to Sb(III) with somewhat higher affinity but with the same stoichiometry as As(III) (Fig. 1D). Mutational analyses previously showed that C132, C137, and C274 are critical for Yap8 function and regulation (12, 21, 22). To test whether these cysteines are also involved in metalloid binding, we purified a mutant version of MBP-Yap8 in which Cys132 and Cys274 were replaced by alanine. Importantly, purified MBP-Yap8-C132A/C274A had lost the majority of the binding to both As(III) and Sb(III) (Fig. 1C and D). Thus, Cys132 and Cys274 are likely part of the three-coordinate As(III)/Sb(III) binding site.

As(III) binds directly to Yap8 *in vivo*. To assess whether arsenic binds directly to Yap8 also *in vivo*, we expressed N-terminal fusions of Yap8 and Yap8-C132A/C274A to glutathione *S*-transferase (GST) in *E. coli* cells and incubated the extracts with an arsenite-biotin conjugate probe (As-biotin) (Fig. 2A) followed by pulldown with streptavidin-agarose beads and anti-GST immunoblotting. The in-solution pulldown assays showed that As-biotin bound to GST-Yap8 (Fig. 2B, lanes 1 and 3) and that binding

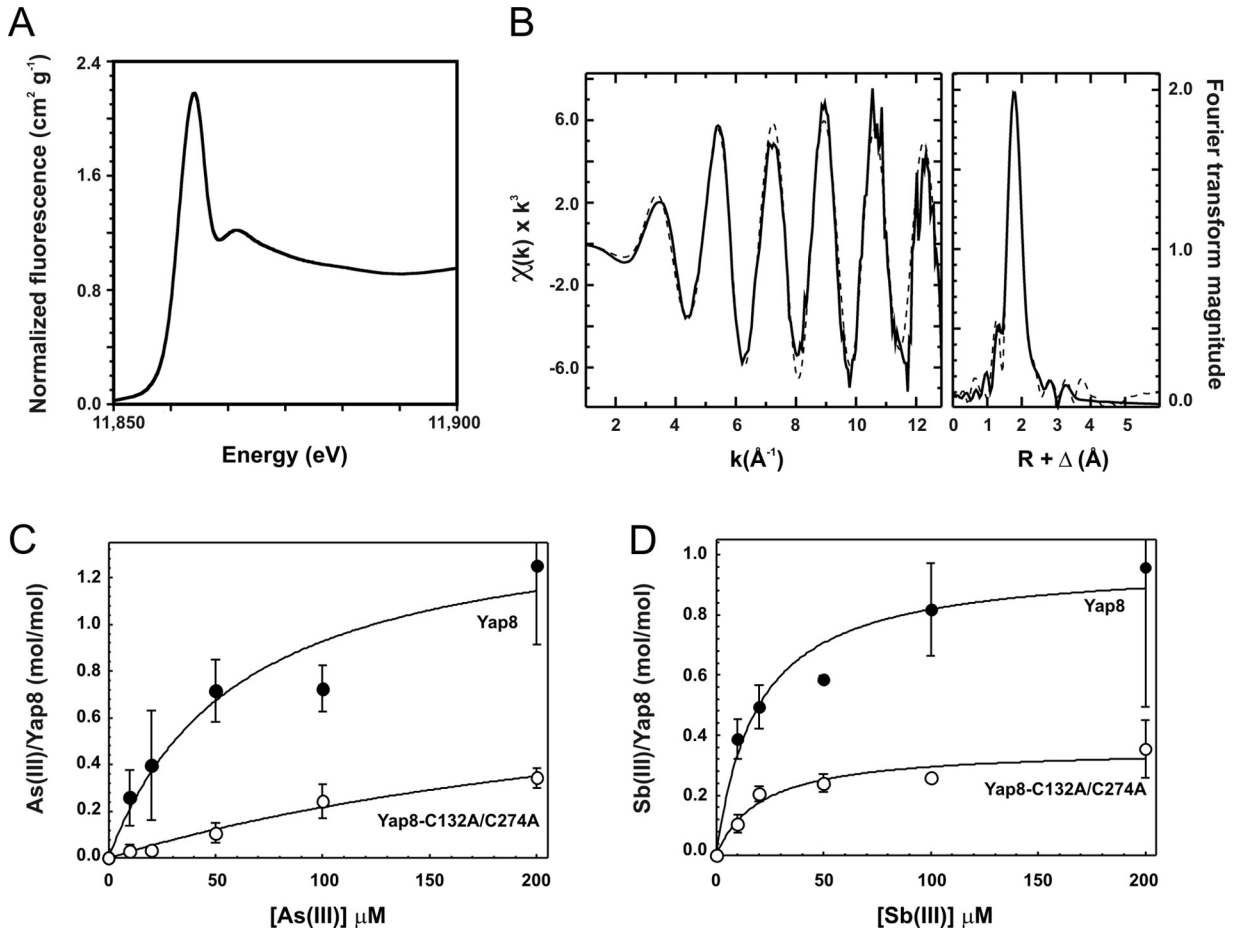

FIG 1 Arsenic binds directly to Yap8 *in vitro*. (A) Normalized XANES spectra for MBP-Yap8 wild-type protein. (B) MBP-Yap8-arsenic EXAFS data and Fourier transforms and simulations. (Left) Raw EXAFS data of As(III) bound to MBP-Yap8 (solid lines) and best-fit simulated data (dashed lines). (Right) Phase-shifted Fourier transforms of the protein-arsenic EXAFS data are in solid lines, and simulated spectra are in dashed lines. (C and D) Stoichiometry of As(III) (C) or Sb(III) (D) binding to purified MBP-Yap8. Binding of As(III) or Sb(III) to wild-type MBP-Yap8 or MBP-Yap8-C132A/C274A was determined after incubating 10 μM purified protein with the indicated concentrations of sodium arsenite or potassium antimonyl tartrate. Free metalloid was removed by gel filtration, and total arsenic or antimony was determined by ICP-MS, as described in Materials and Methods. The lines represent the best fit of the data using SigmaPlot 9.0. The error bars represent standard deviations ($n = 3$).

was attenuated when the bacterial lysate was pretreated with an equal concentration of As(III) (Fig. 2B, lanes 3 and 7). Compared to the wild-type GST-Yap8 protein, GST–Yap8-C132A/C274A showed dramatically reduced binding to As-biotin (Fig. 2B, compare lanes 3 and 4). The results from the pulldown assays are consistent with the finding that MBP-Yap8-C132A/C274A had lost most of its metalloid binding capacity *in vitro* (Fig. 1C and D).

TABLE 1 Summary of best-fit parameters from the MBP-Yap8-As(III) EXAFS fitting analysis[a]

Atom	R (Å)	CN	σ^2
S	2.24	3.0	2.5
C	2.70	0.5	4.5
C	3.69	2.0	4.7

[a] Data were fit over a k range of 1 to 12.85 $Å^{-1}$. Abbreviations: R, average metal-ligand bond length from three independent samples; CN, average metal-ligand coordination number from three independent samples; σ^2, average Debye-Waller factor (in 10^3 $Å^2$) from three independent samples. The overall goodness of fit parameter, weighted for the number of degrees of freedom within the multicomponent simulation, F′, is equal to 0.81.

The ability of As(III) to bind to Yap8 present in bacterial cell lysates strongly suggests that a direct interaction occurs between the cysteines in Yap8 and the metalloid *in vivo* and that no additional yeast protein is involved in this process.

We next assessed binding of arsenic to Yap8 in *S. cerevisiae* cells. For this, we expressed c-myc-tagged versions of Yap8 (myc-Yap8 and myc-Yap8-C132A/C274A) in *yap8*Δ cells and treated the yeast extracts with the As-biotin conjugate as above. Importantly, the pulldown assays demonstrated that As-biotin efficiently bound to myc-Yap8 (Fig. 2C, lanes 1 and 3), whereas myc-Yap8-C132A/C274A had lost most of its As(III) binding capacity (Fig. 2C, compare lanes 3 and 4). Moreover, As-biotin binding to myc-Yap8 was attenuated when the yeast lysates were pretreated with an equal concentration of As(III) (Fig. 2C, lanes 3 and 7). These data strengthen the conclusion that a direct interaction occurs between the cysteines in Yap8 and As(III) also in yeast.

The Yap8 orthologue from *Kluyveromyces lactis* binds to As(III). The milk yeast *K. lactis* possesses a *YAP8* orthologue (*KlYAP8*). *Kl*Yap8 regulates As(III)-dependent gene expression and confers As(III) tolerance in *K. lactis*. Moreover, *Kl*Yap8 can

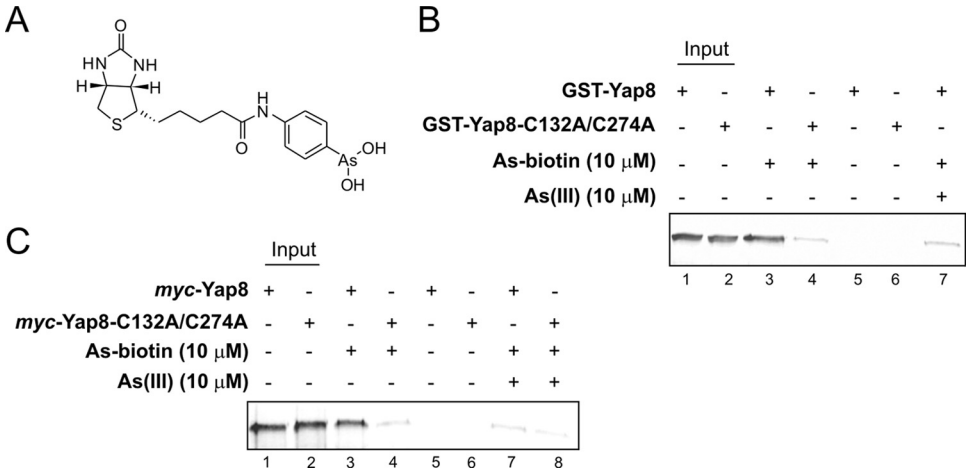

FIG 2 Arsenic binds directly to Yap8 *in vivo*. (A) Structure of the As-biotin conjugate used. (B) As-biotin binds to GST-Yap8 in *E. coli* cell lysates. *E. coli* cell lysates expressing GST-Yap8 or GST–Yap8-C132A/C274A were incubated with 10 μM As-biotin conjugate followed by pulldown with streptavidin-agarose beads and anti-GST immunoblotting. The GST-Yap8 lysate was pretreated with 10 μM As(III) for 30 min prior to the addition of As-biotin conjugate (last lane). Lanes 1 and 2 represent the input. (C) As-biotin binds to myc-Yap8 in *S. cerevisiae* cell lysates. *S. cerevisiae* cell lysates expressing myc-Yap8 or myc-Yap8-C132A/C274A were incubated with 10 μM As-biotin conjugate followed by pulldown with streptavidin-agarose beads and anti-c-myc immunoblotting. Where indicated, lysates were pretreated with 10 μM As(III) for 30 min prior to the addition of As-biotin conjugate. Lanes 1 and 2 represent the input.

functionally replace *S. cerevisiae* Yap8 (35). The *Kl*Yap8 protein has 10 cysteines, 3 of which correspond to the critical cysteines Cys132, Cys137, and Cys274 in *S. cerevisiae* Yap8 (Fig. 3A). Like for *S. cerevisiae* Yap8, As-biotin bound to GST-*Kl*Yap8 in the in-solution pulldown assay (Fig. 3B, lanes 1 and 2), and this binding was attenuated when the bacterial lysates were pretreated with an equal concentration of As(III) (Fig. 3B, lanes 2 and 5). Thus, the ability to bind As(III) appears to be evolutionarily conserved in Yap8-like proteins.

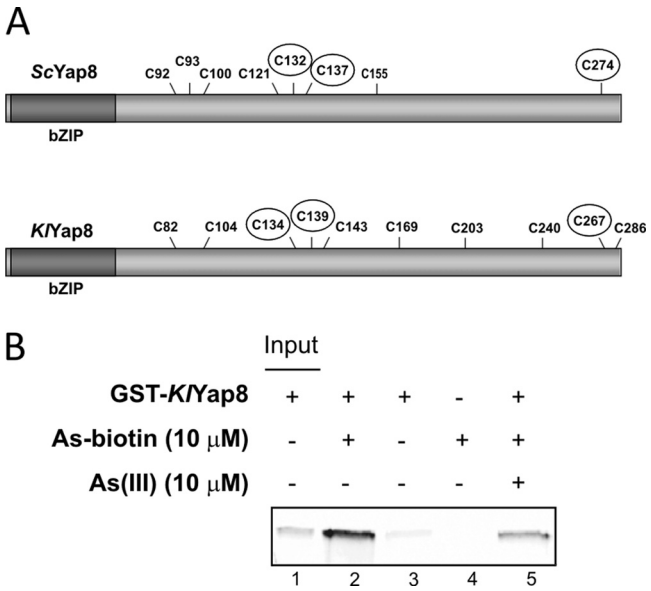

FIG 3 Arsenic binds to *K. lactis* Yap8. (A) Schematic representation of Yap8 from *S. cerevisiae* and *K. lactis* (*Sc*Yap8 and *Kl*Yap8, respectively). The positions of cysteine residues are indicated, with conserved cysteines circled. bZIP, basic leucine zipper. (B) As-biotin binds to GST-*Kl*Yap8 in *E. coli* cell lysates. *E. coli* cell lysates expressing GST-*Kl*Yap8 were incubated with 10 μM As-biotin conjugate followed by pulldown with streptavidin-agarose beads and anti-GST immunoblotting. The GST-*Kl*Yap8 lysate was pretreated with 10 μM As(III) for 30 min prior to the addition of As-biotin conjugate (lane 5). Lane 1 represents the input.

As(III) binding does not affect Yap8 localization or DNA association. To investigate how As(III) binding regulates Yap8, we first monitored subcellular localization of Yap8 and Yap8-C132A/C274A fused to green fluorescence protein (GFP) in *S. cerevisiae*. Fluorescence microscopy showed that GFP-Yap8 and GFP–Yap8-C132A/C274A were localized in the nucleus of living yeast cells, both in the absence and presence of As(III) (Fig. 4A). This is consistent with our previous finding that Yap8 is constitutively nuclear (12). Thus, As(III) binding to Yap8 does not affect its localization.

We next tested the capacity of purified MBP-Yap8 and MBP-Yap8-C132A/C274A to bind to the *ACR2*/*ACR3* promoter. The results of *in vitro* DNA binding studies by fluorescence anisotropy of a fluorescein (FAM)-labeled DNA oligonucleotide indicated that purified MBP-Yap8 binds to its cognate promoter site (Fig. 4B). Addition of up to 5 mM As(III) did not have any significant effect on the binding of MBP-Yap8 to DNA. Moreover, MBP-Yap8-C132A/C274A, which lacks the As(III)-binding cysteines, retained full capability of DNA binding (Fig. 4B). We also examined DNA occupancy of myc-Yap8 and myc-Yap8-C132A/C274A in yeast. Chromatin immunoprecipitation assays demonstrated that As(III) exposure does not affect myc-Yap8 occupancy on the *ACR3* promoter *in vivo* (Fig. 4C). DNA occupancy of myc-Yap8-C132A/C274A appeared somewhat higher than for myc-Yap8 and was largely unaffected by As(III) exposure. Together, these data strongly suggest that the DNA binding activity of Yap8 is As(III) independent.

As(III) binding induces a conformational change in Yap8. Since As(III) binds to Yap8 and Yap8 is stabilized in As(III)-exposed yeast cells (21), we hypothesized that metalloid binding might affect Yap8 conformation. As(III) binding to the ArsA ATPase, the catalytic subunit of the *E. coli* As(III) efflux pump, produces conformational changes that confer protection to limited trypsin digestion (37). To probe Yap8 for As(III)-dependent

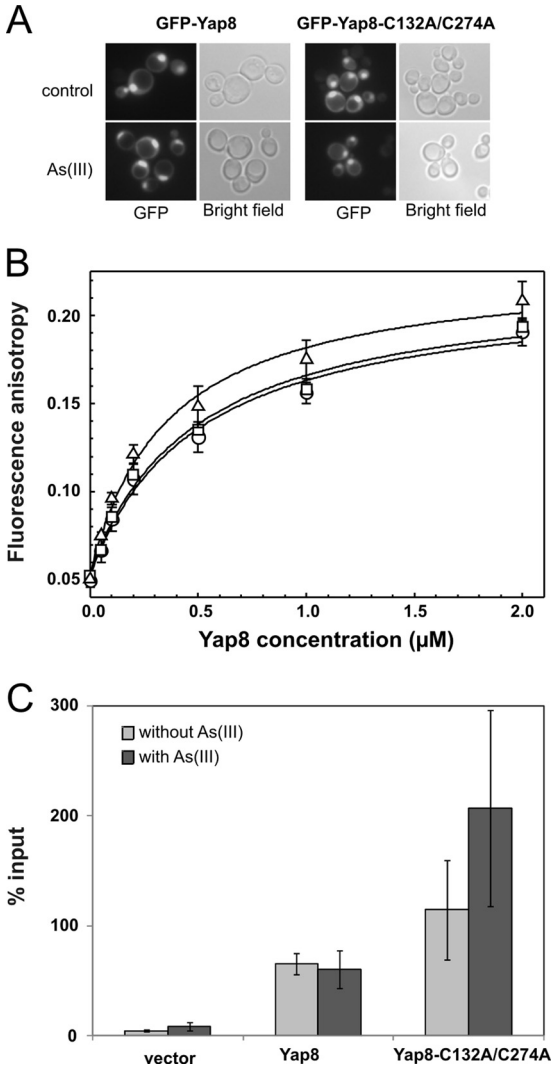

FIG 4 Yap8 is not regulated at the level of localization or DNA binding. (A) Yap8 localization. Plasmids carrying GFP fusions of Yap8 and Yap8-C132A/C274A were transformed into *yap8*Δ cells, and the proteins were visualized with fluorescence microscopy in the absence (control) and presence of As(III). (B) *In vitro* Yap8-DNA binding. Binding of purified MBP-Yap8 to promoter DNA was estimated from the increase in fluorescence anisotropy, as described in Materials and Methods. Fluorescently labeled double-stranded DNA was titrated with the indicated concentrations of wild-type MBP-Yap8 in the absence (○) or presence (□) of 5 mM As(III) or with MBP-Yap8-C132A/C274A (Δ). The lines represent the best fit of the data using SigmaPlot 9.0. The error bars represent standard deviations ($n = 3$). (C) *In vivo* occupancy of myc-Yap8 and myc-Yap8-C132A/C274A on the *ACR3* promoter as determined by ChIP. Plasmids carrying myc-Yap8 or myc-Yap8-C132A/C274A were transformed into *yap8*Δ cells together with an empty vector as a control. The transformants were either untreated or exposed to 0.5 mM As(III) for 1 h, and qPCR was performed on chromatin fragments isolated after immunoprecipitation (anti-c-myc-IP) as described in Materials and Methods. Data from 3 biological replicates are shown, and the error bars represent standard deviations.

conformational changes, we performed a similar trypsin susceptibility assay. Total cell extracts were prepared from *E. coli* cells expressing GST-Yap8 that had been either untreated or exposed to As(III). The cell lysates were then subjected to limited trypsin proteolysis followed by anti-GST immunoblotting to visualize trypsin digestion patterns. Importantly, GST-Yap8 was found to be more resistant to trypsin digestion when *E. coli* cells were exposed to As(III) (Fig. 5A, upper panel). In contrast, GST–Yap8-C132A/C274A, which lacks the As(III)-binding cysteines, was not protected from trypsin proteolysis when *E. coli* cells were treated with As(III) (Fig. 5A, lower panel).

We next expressed myc-Yap8 and myc-Yap8-C132A/C274A in *S. cerevisiae* and exposed the yeast cells to As(III). Immunoblotting showed that myc-Yap8 is stabilized in As(III)-exposed cells whereas myc-Yap8-C132A/C274A is not (Fig. 5B), confirming that Yap8 is protected from ubiquitin-dependent proteolysis *in vivo* (21). Importantly, myc-Yap8 was clearly more resistant to *in vitro* trypsin digestion when the protein was extracted from yeast cells cultured in the presence of As(III) (Fig. 5B, upper panel). In contrast, As(III) did not protect myc-Yap8-C132A/C274A from trypsin proteolysis (Fig. 5B, lower panel). Quantifications of remaining GST-Yap8 (Fig. 5C) and myc-Yap8 (Fig. 5D) levels after trypsin digestion (from two biological replicates each) support the notion that Yap8 is more resistant to *in vitro* proteolysis when extracted from As(III)-exposed cells. Note that trypsin proteolysis of Yap8-C132A/C274A was similar when the protein was extracted from cells cultured with and without As(III) (Fig. 5). Thus, protection from trypsin cleavage is selective for Yap8. This indicates that As(III) binding to the protein rather than the presence of traces of As(III) in the protein extract is a determining factor of the cleavage, eliminating the possibility that trypsin itself is inactivated by As(III). Instead, our data indicate that As(III) binding *in vivo* induces a conformational change in Yap8 that protects the protein from cleavage.

As(III) binding to Yap8 is required for *ACR2*/*ACR3* expression and for arsenic tolerance. We next monitored growth and *ACR2*/*ACR3* expression in As(III)-exposed yeast cells. *S. cerevisiae* harboring GFP-Yap8 efficiently induced expression of *ACR2* and *ACR3* in response to As(III) treatment (Fig. 6A). Moreover, these cells grew well in the presence of As(III) (Fig. 6B). In contrast, cells that harbored GFP–Yap8-C132A/C274A, which cannot bind to As(III), were clearly defective in As(III)-induced *ACR2* and *ACR3* expression and displayed As(III) sensitivity (Fig. 6A and B). These data indicate that As(III) binding to Yap8 is required for induction of *ACR2*/*ACR3* expression and for arsenic tolerance.

DISCUSSION

Based on the abundance of arsenic in the environment, its toxicity, and the potential for human exposure, the U.S. Agency for Toxic Substances and Disease Registry ranks arsenic first on the U.S. Priority List of Hazardous Substances (38). Long-term exposure to this toxic metalloid can cause a range of human diseases, including cancer, diabetes, and cardiovascular disorders. In addition, arsenic has a long history of usage as a chemotherapeutic agent and is an important constituent of currently used pharmacological drugs. The impact of arsenic on the environment and human health underscores the importance of elucidating the mechanisms by which cells detect and cope with this metalloid (1, 8, 39, 40). The ability to sense the presence of toxic agents and to regulate tolerance and detoxification systems is essential for cell viability. While prokaryotic arsenic-sensing proteins have been described, little is known about arsenic sensing in eukaryotic organisms. Here we demonstrate that the yeast transcription factor Yap8 senses As(III) by directly binding to this metalloid. XANES and EXAFS analyses revealed that As(III) is coordinated by three sulfur-based ligands when it binds to MBP-Yap8, indicating that

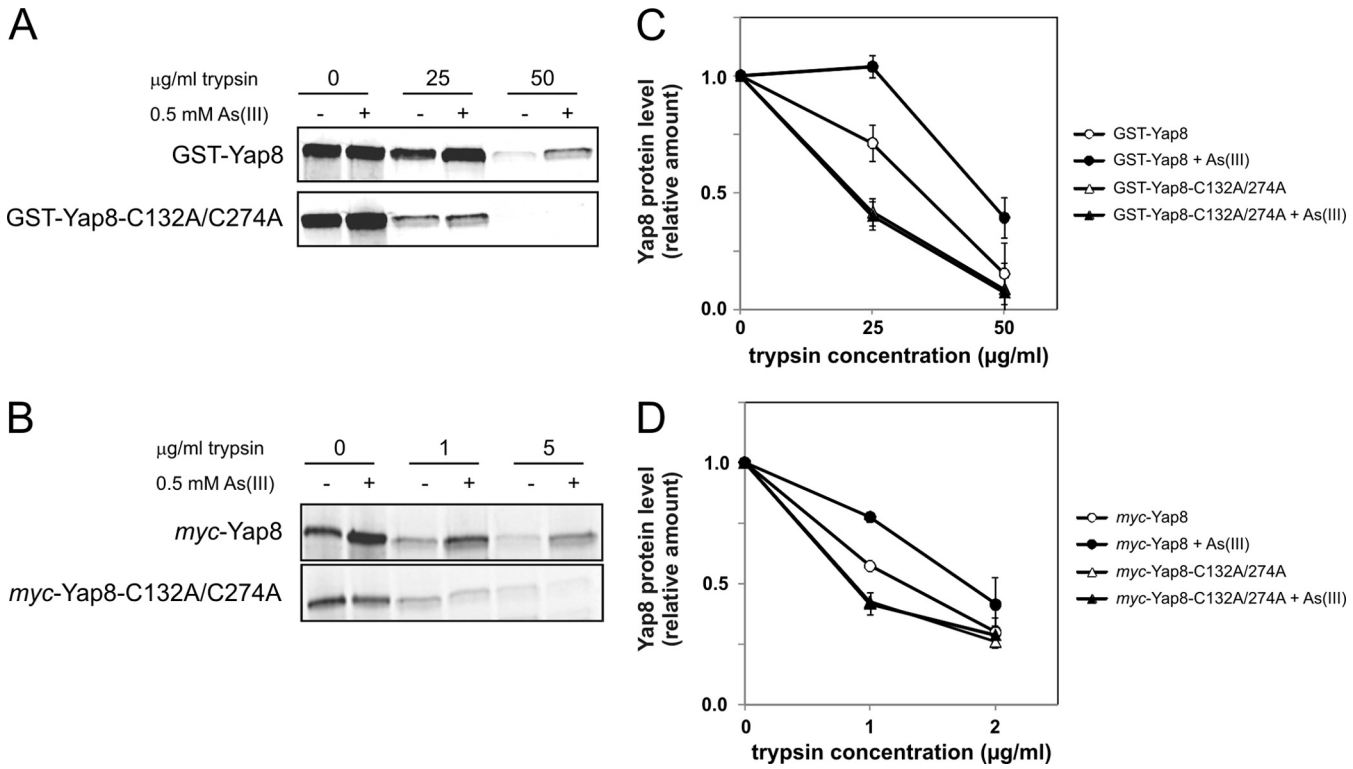

FIG 5 As(III) binding induces a conformational change in Yap8. (A) *E. coli* cell lysates prepared from cells that were either untreated or exposed to 0.5 mM As(III) for 45 min were subjected to increasing concentrations of trypsin, and proteolysis patterns were visualized using an anti-GST antibody. (B) *S. cerevisiae* cell lysates prepared from cells that were either untreated or exposed to 0.5 mM As(III) for 1 h were subjected to increasing concentrations of trypsin, and proteolysis patterns were visualized using an anti-c-myc antibody. (C and D) Quantification of GST-Yap8 (C) and myc-Yap8 (D) protein levels. Protein levels in the absence of trypsin were set to 1, and the remaining levels after trypsinization were related to that before cleavage. The error bars represent standard deviations from two independent biological replicates each ($n = 2$).

the metalloid binding site is formed by three cysteines (Fig. 1; Table 1). When Cys132 and Cys274 were mutated to alanine, the As(III) binding ability of Yap8 was dramatically disrupted, both *in vitro* (Fig. 1) and *in vivo* (Fig. 2). Likewise, Cys132 and Cys274 were also critical for Sb(III) binding (Fig. 1). Thus, Cys132 and Cys274 are part of the metalloid binding site in Yap8. The third cysteine forming the As(III)/Sb(III) binding site is probably Cys137; first, mutation of any of the cysteines Cys132, Cys137, and Cys274 individually or mutation of Cys132 and Cys274 together resulted in the same inability of Yap8 to induce *ACR3* expression and to confer arsenic tolerance (12, 21); second, each of these cysteines are equally important for As(III)-induced Yap8 stabilization (21); third, Cys132, Cys137, and Cys274 are conserved in *K. lactis Kl*Yap8 (Fig. 3) and in *S. cerevisiae* Yap1 (12). Collectively, our genetic, molecular, and biochemical data provide strong evidence that Cys132, Cys137, and Cys274 form the metalloid binding site in *S. cerevisiae* Yap8. While Cys274 is separated by ~140 amino acids from Cys132 and Cys137, EXAFS analyses indicated that the three cysteines are located 2.24 Å from each other when As(III) is bound (Fig. 1; Table 1). Whether these residues are close to each other also when Yap8 is inactive or are brought together to allow As(III) binding remains to be determined. In either case, As(III) binding to Yap8 does not require an additional yeast protein *in vivo* (Fig. 2). We conclude that Yap8 directly senses the presence of As(III) in the cell.

How does As(III) binding regulate Yap8? Our data indicate that Yap8 is not regulated at the level of nuclear localization and that the DNA binding activity of Yap8 is As(III) independent (Fig. 4). Instead, we show using *in vitro* trypsin proteolysis assays that Yap8 undergoes a conformational change upon As(III) binding (Fig. 5). The Cys132A/Cys274A form of Yap8, which cannot efficiently bind to As(III), did not go through a conformational change (Fig. 5), was defective in activation of *ACR2*/*ACR3* expression, and was unable to confer arsenic tolerance (Fig. 6). The finding that As(III) binding causes a conformational change in Yap8 is consistent with the requirement of Cys132, Cys137, and Cys274 for Yap8 to escape ubiquitin-dependent degradation during As(III) exposure *in vivo* (21) (Fig. 5B), since an As(III)-dependent conformational change may mask a degradation signal in Yap8 resulting in its stabilization. This conformational change may also facilitate the recruitment of RNA polymerase II to the *ACR2*/*ACR3* promoter, leading to enhanced gene expression. We note that Yap8 and Yap8-C132A/C274A bound similarly to the *ACR3* promoter both in the absence and in the presence of As(III) (Fig. 4B and C), whereas only Yap8 is protected from proteolysis in the presence of As(III) (Fig. 5B) (21). Thus, the relative abundance of Yap8 seems to play a minor role in promoter occupancy and gene activation. Taken together, our data are consistent with a model in which a DNA-bound version of Yap8 acts directly as an As(III) sensor. The binding of As(III) to Yap8 triggers a conformational change that in turn brings about a transcriptional response. Thus, As(III) binding to Yap8 may act as a molecular switch that con-

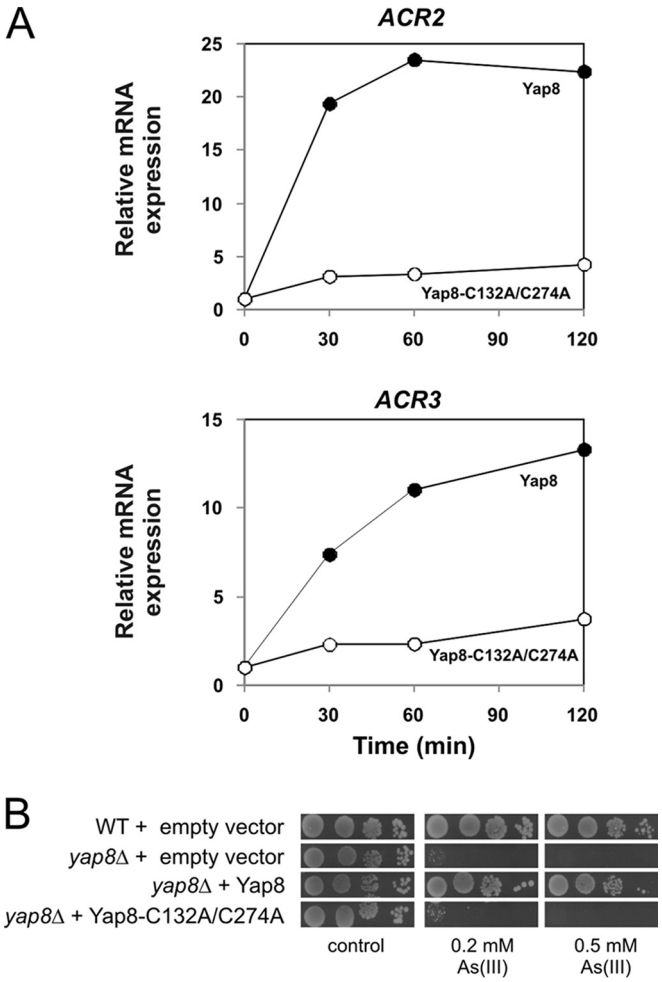

FIG 6 Yap8 is required for expression of arsenic resistance genes. (A) Gene expression. qPCR results of *S. cerevisiae yap8*Δ cells transformed with plasmids containing either GFP-Yap8 or GFP–Yap8-C132A/C274A. Transformants were exposed to 0.5 mM As(III), and samples for RNA extraction were taken at the indicated time points. *ACR2* and *ACR3* expression was normalized to *IPP1* expression. Values are the means of two biological replicates performed in triplicate. (B) Growth analysis. Wild-type and *yap8*Δ cells were transformed with the indicated plasmids, spotted onto plates with or without As(III), and incubated for 2 days at 30°C.

verts inactive Yap8 into an active transcriptional regulator (Fig. 7). This mechanism is different from that of *E. coli* ArsR, which is a negative regulatory protein that needs to dissociate from the promoter to derepress gene expression.

How does Yap8 activate transcription? Transcription factors can regulate transcription by recruiting activation or repression complexes through protein-protein interactions. For example, binding of zinc to the yeast transcription factor Zap1 results in a conformational change that inhibits Zap1's ability to recruit coactivators to target promoters (41–43). An alternative mechanism of gene regulation was recently elucidated for the copper-sensing transcription factor CueR in *E. coli*. CueR was found to repress or activate transcription while it remained bound to exactly the same promoter sequence. Upon copper binding, CueR introduces changes in the topology of the promoter, which in turn allows binding of RNA polymerase to the promoter (44). Whether Yap8 activates transcription by protein-protein interactions or by DNA topology changes remains to be determined.

How do other yeast AP-1-like transcription factors sense As(III)? We show here that *Kl*Yap8 from *K. lactis* directly binds to As(III) (Fig. 3). This binding may involve Cys134, Cys139, and Cys267 in *Kl*Yap8, since these residues correspond to the critical cysteine triad in *S. cerevisiae* Yap8 (Fig. 3). Thus, it is likely that the As(III)-sensing mechanism is similar in Yap8 proteins across different genera. Interestingly, while *S. cerevisiae* Yap8 is activated only by As(III) and Sb(III) (12), *Kl*Yap8 appears to be a sensor of As(III), peroxide, and cadmium (35). How *Kl*Yap8 discriminates between these signals is currently unknown. *S. cerevisiae* Yap1 accumulates in the nucleus and activates gene expression in response to a range of oxidants, metals, and the metalloids As(III) and Sb(III). These stress signals activate Yap1 through distinct mechanisms that involve disulfide bond formation and/or covalent modifications of conserved cysteines (2, 45). Mutation of Yap1 cysteines that correspond to the critical cysteine triad in Yap8 resulted in perturbed nuclear accumulation of Yap1 and a diminished ability of the transcription factor to confer As(III) tolerance (12). Whether As(III) sensing by Yap1 involves a direct interaction between these cysteines and the metalloid or whether As(III) activates Yap1 through oxidative modifications remains to be elucidated.

To conclude, this is the first report to demonstrate how a eu-

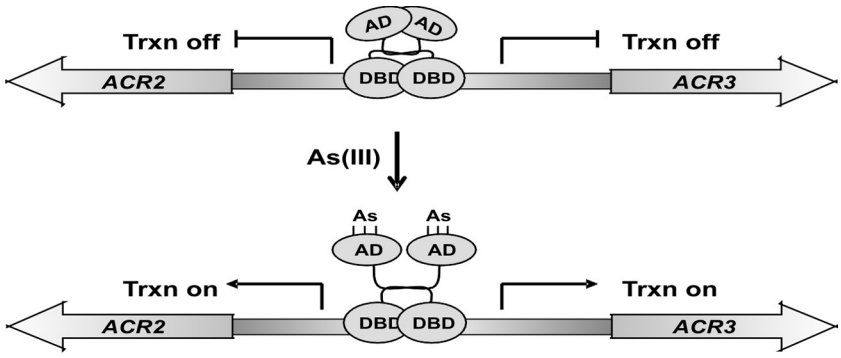

FIG 7 Model of As(III) sensing and transcriptional activation (Trxn) by Yap8. Our data are consistent with a model in which a DNA-bound version of Yap8 acts directly as an As(III) sensor *in vivo*. Nuclear Yap8 binds to the *ACR2-ACR3* promoter as a homodimer. In the absence of As(III), Yap8 shows no transcriptional activity. Activation of Yap8 involves direct binding of As(III) to the cysteine residues Cys132, Cys137, and Cys274. Binding of As(III) to Yap8 triggers a conformational change that results in induced *ACR2* and *ACR3* expression. Thus, As(III) binding to Yap8 may act as a molecular switch that converts inactive Yap8 into an active transcriptional regulator. AD, activation domain; DBD, DNA binding domain.

karyotic protein couples arsenic sensing to transcriptional activation. Therefore, Yap8 may serve as a model for how higher organisms sense and respond to environmental arsenic. Interestingly, several yeast AP-1 factors can sense and respond to oxidative stress, metals, and/or metalloids, and the sensing mechanisms involve cysteine residues. Future comparative analyses of *S. cerevisiae* Yap8, *S. cerevisiae* Yap1, and *K. lactis Kl*Yap8 may shed light on whether the functional metalloid binding site of Yap8 evolved from an ancestral AP-1 protein by adapting a site for sensing oxidative stress into a three-coordinate site for As(III)/Sb(III), thereby providing insights into the evolution of metalloid binding sites in sensor proteins.

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